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(71) Applicant
Kureha Kagaku Kogyo Kabushiki Kaisha, 9—11, Horidome-cho, 1-chome, Nihonbashi, Chuo-ku, Tokyo, Japan
(72) Inventors
Takao Ando,
Norio Takahashi,
Tohru Hirai,
Chikao Yoshikumi,
Masahiko Fujii,
Kenichi Matsunaga,
Yoshiharu Oguchi
(74) Agents
J. A. Kemp & Co.,
14, South Square, Gray's Inn, London, WC1R 5EU

(54) Production of Glycoprotein having Immunosuppressive Activity

(57) A glycoprotein possessing immunosuppressive activity is obtained by subjecting the serum or the ascites of a human or warm-blooded animal to isoelectric focussing and collecting the fraction at an isoelectric point in the range of 2.6 to 3.6. This glycoprotein can be used to suppress the immune response in transplant patients.

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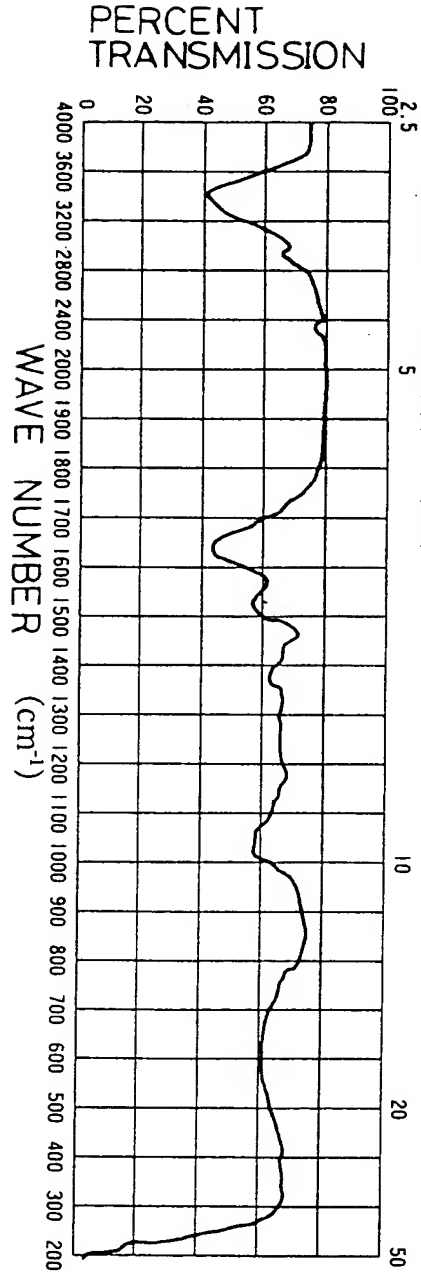
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60
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WAVE NUMBER (cm⁻¹)
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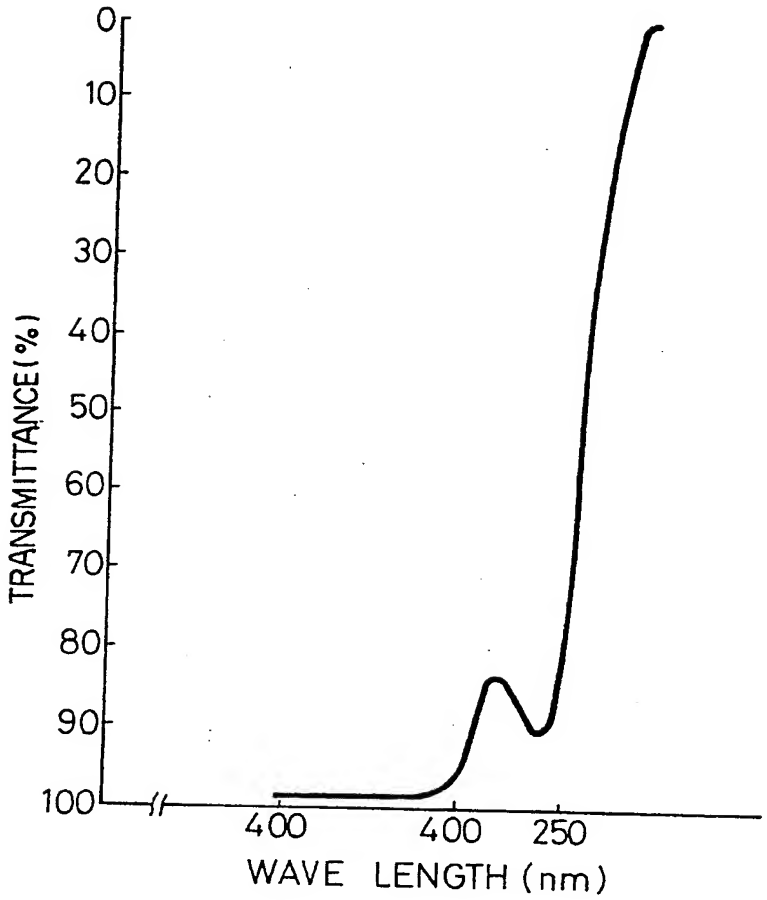
WAVE LENGTH (μm)
2.5
5
10
20
50

FIG. 1

FIG. 2


0
10
20
30
40
50
60
70
80
90
100
TRANSMITTANCE (%)
400
400
250
WAVE LENGTH (nm)

SPECIFICATION

Production of Glycoprotein Having Immunosuppressive Activity

The present invention relates to a method for preparing a glycoprotein having an immunosuppressive activity from the serum or ascites of human or warm-blooded animals, and the glycoprotein thus obtained.

The characteristic feature of the present invention is to obtain a glycoprotein having an immunosuppressive activity by subjecting the serum or ascites of human or warm-blooded animals to the isoelectric focussing and thus collecting the fraction at an isoelectric point in the range of 2.6 to 3.6.

In Drawings, Fig. 1 shows the infrared absorption spectrum of the glycoprotein obtained according to the present invention, and Fig. 2 shows the ultraviolet absorption spectrum of the glycoprotein.

Hitherto, in the diagnosis of cancers and the estimation of the degree of progress of cancerous diseases, the determination of a foetal protein appearing in the cancer patient's serum such as alpha-fetoprotein and carcinoembryonic antigen (CEA), determination of the sensitivity of the patient to tuberculin (PPD) and to 2,4-dinitrochlorobenzene, and determination of the degree of blastogenesis of lymphocytes in the patient's peripheral blood due to phytohemagglutinine have been broadly applied in the clinical field of cancerology.

These determinations and the test reactions are carried out based on the correlation between the reduction of the cancer patient's immunity and the progress of the patient's cancerous disease, and in recent years, there appear reports concerning the presence of substances having an immunosuppressive activity of human and warm-blooded animals in the serum of human and warm-blooded animals. For example, Cooperband et al. reported immuno-regulatory alpha-globulin as the above-mentioned substance (refer to Transplantation Proceedings, Vol. 8, No. 2 (1976) pp 225—242) and Ishida et al. reported on acidic protein as the above-mentioned substance (XXXV Annual Meeting of Japanese Cancer Association, 1976).

These substances having an immunosuppressive activity suppress the cellular immunity and the humoral immunity of human and warm-blooded animals and thus suppress the graft rejection. Accordingly, such a substance is useful in preventing and treating the rejection in the cases of transplantation.

According to Ishida et al., the acidic protein having an immunosuppressive activity is prepared by subjecting the serum or ascites of human or warm-blooded animals to anion-exchange chromatography and collecting the fraction which is eluted at a pH in a range of 2.9 to 3.3 (refer to Japanese Patent Application Laying open No. 53-44611/1978, being published on November 6, 1980 as No. 55-43479/1980). However, the acidic protein reported by Ishida et al, is fractionally collected by adsorption and elution of the serum or ascites, and the method has a demerit of giving a relatively small amount of the acidic protein of a relatively low activity of suppressing immunity because of the practically intricate steps.

The present inventor, as a result of studying the simplified method of effectively obtaining a glycoprotein of a high activity of suppressing immunity from the serum or ascites of human or warm-blooded animals in a large amount, has attained the present invention.

Accordingly, the main purpose of the present invention is to provide a glycoprotein having a high immunosuppressive activity in human or warm-blooded animals and a method for preparing the glycoprotein from the serum or ascites of human or warm-blooded animals in a large amount. Other objects of the present invention will be made clear from the following description.

The present invention will be explained more in detail in the following:

As a starting material for use in the present invention, the serum or ascites of a cancer patient or of a warm-blooded animal to which cancer cells or cancer tissue have been transplanted can be exemplified.

In order to subject the serum or ascites to isoelectric focussing, for example, in the case where the ascites of a cancer patient is used, the ascites is centrifuged or filtered to remove the solid matters not dissolved therein and then the cleaned ascites is subjected to the following column isoelectric focussing or the following granular gel isoelectric focussing at a pH in the range of 2.6 to 3.6.

On carrying out the column isoelectric focussing, an amphoteric carrier of pH of 2.5 to 6.0 such as Ampholine (manufactured by LKB Company, Sweden) is dissolved in water, and the solution and an aqueous solution of sucrose, ethylene glycol or glycerol were used to prepare a solution having a density gradient in a glass tube. In such a kind of column, the cleaned serum or ascites is subjected to isoelectric focussing at a constant power from 3 W to 20 W and then, the fraction at an isoelectric point of pH of 2.6 to 3.6 was collected.

In case of the granular gel isoelectric focussing, a plate is prepared by Ampholine and a gel substance such as Sephadex G-75 (manufactured by Pharmacia Company, Sweden), and the cleaned serum or ascites is fractioned after isoelectric focussing on the plate.

The amphoteric substance and the substance for making density gradient, which still remain in the collected fraction are removed by subjecting the fraction to dialysis or ultrafiltration to purify the fraction.

The thus obtained fraction of electrophoresis is freeze-dried to be the object, the glycoprotein.

The glycoprotein obtained according to the

present invention has the following physical properties:

1. Appearance: white powder,
2. Solubility: Soluble in water, aqueous physiological saline solution and phosphoric acid-buffer solution, and Insoluble in methanol, ethanol, propanol, butanol, acetone, chloroform, ether and ethyl acetate,
3. Isoelectric point: 2.6 to 3.6 of pH (determined by isoelectric focussing in a flat plate),
4. Colour reactions: Positive in Lowry-Folin's reaction, Ehrlich's reaction, Buret's reaction, ninhydrin reaction and Molisch's reaction (phenolsulfuric acid reaction),
5. Infrared absorption spectrum: as shown in Fig. 1, by KBrmethod, the concentration being 0.2% by weight,
6. Ultraviolet absorption spectrum: as shown in Fig. 2, in pure aqueous solution at a concentration of 0.01%.

The activity of the present substance in suppressing immunity is explained as follows:

According to the result of investigation of the immunosuppressive activity of the present substance on the suppression of blastogenesis of lymphocytes in human peripheral blood and mouse splenic cells by phytohemagglutinin (PHA), 1.0 mg of the present substance in one millilitre showed a suppression of 100% of the former and of 90% of the latter, and even 0.1 mg of the present substance in one millilitre showed a suppression of 80% of the former and 55% of the latter.

According to the result of investigation of delayed-type of foot-pad reaction against sheep erythrocyte in CIR mouse, the intraperitoneal administration of 100 microgram per animal suppressed the reaction about 50% as compared to control not administered with the present substance.

From the above-mentioned results, it is understood that the present substance has a high immunosuppressive activity.

Accordingly, it can be said, that the present substance is useful in preventing and treating the immunological rejection in the case of surgical transplantation.

In order to apply the glycoprotein obtained according to the present invention into the prevention and the treatment of the immunological rejection in the case of operation of transplantation, the glycoprotein is administered by intraperitoneal injection before about 24 hours of the operation and the intraperitoneal administration of the glycoprotein is further carried out after the operation. The dose is generally 20 mg/kg/day once before the operation and the same amount was administered for continuous 14 days after the operation.

In addition, the acute toxicity of the glycoprotein was examined by the following test:

A series of aqueous solution of the glycoprotein were intraperitoneally injected into 15 mice of DKI to see their mortality for a week after the injection. Even at the highest concentration corresponding to 1000 mg/kg of body weight, no death was observed among the treated mice.

The present invention will be concretely explained while referring to examples as follows:

Example 1

After leaving 10 ml of the blood taken from a patient of hepatic cancer as it is for 30 to 60 min, it was subjected to centrifugation at about 1500 G for 10 min to obtain 5 ml of a supernatant liquid.

Separately, two aqueous solutions of Ampholine (loc. cit.) of the respective pH of 2.5 to 4.0 and of 4.0 to 6.0 were mixed together to be an aqueous solution of amphoteric carrier of pH of 2.6 to 6.0 (herein after referred to as Ampholine Solution).

Then, two kinds of liquid mixture, the Dense liquid (1) and the Light liquid (2) were prepared: (1) by mixing 11 ml of Ampholine Solution with 137 ml of distilled water containing 108 g of sucrose and (2) by mixing 3.6 ml of Ampholine Solution and a solution prepared by dissolving 5 ml of the supernatant liquid of the specimen (blood) into distilled water to be a solution of 211 ml and further dissolving 10.8 g of sucrose into the solution. These two kinds of liquid mixture were filled into a glass tube of capacity of 440 ml (Glass Column for isoelectric focussing, type 8102, manufactured by LKB Company, Sweden) after mixing together in a specified "gradient" mixer. The isoelectric focussing was begun at a voltage of 550 V, a current of 9.1 mA and continued at a constant power of 5 W for 23 hours and was stopped at a voltage of 1500 V.

After the isoelectric focussing was over, the fraction between pH of 2.6 and 3.6 was collected from the Glass Column, subjected to dialysis against a flowing water to remove Ampholine and sucrose still remaining in the fraction and subjected to freeze-drying to obtain the purified glycoprotein in an amount of 5.2 mg per 10 ml of the specimen (blood).

According to the results of examination on the suppression by the thus obtained glycoprotein of the blastogenesis of the lymphocytes in human peripheral blood and of the splenic cells of mouse due to PHA, the degree of suppression of blastogenesis of the lymphocites and the splenic cells was respectively 100 and 90% at a concentration of 1.0 mg of the glycoprotein/ml, and 80 and 55% at a concentration of 0.1 mg of the glycoprotein/ml.

In addition, according to the examination on the suppression by the glycoprotein, intraperitoneally injected into ICR mouse at a rate of 100 μg/animal, of the delayed type footpad reaction due to sheep's erythrocytes, the degree of suppression was 49%.

Example 2

Twenty five milliliters of an ascites collected from a human patient of colonal cancer was

subjected to centrifugation on a cooling centrifuge at 10,000 G for 30 min to obtain a supernatant liquid.

The thus obtained supernatant liquid was subjected to isoelectric focussing in a plate-type isoelectric focussing apparatus (manufactured by LKB Company, Sweden) using a plate prepared in Example 1 from a solution of Ampholine of pH of 2.5 to 6.0 and Sephadex G-75 at a constant power of 8 W for 40 hours and starting at a voltage of 300 V and current of 26.7 mA. After the focussing was reached at a voltage of 1200 V, the fraction of pH of 2.6 to 3.6 was collected together with Sephadex G-75, washed with water to wash out the fraction. The thus obtained liquid was subjected to dialysis against a flowing water to remove Ampholine and the dialyzate was freeze-dried to obtain 24 mg of glycoprotein.

According to the results of examination of the immunosuppressive activity of the thus obtained glycoprotein as in Example 1, the degree of suppression of blastogenesis of the lymphocytes in human peripheral blood due to PHA was 100% at a concentration of 1.0 mg of the glycoprotein/ml.

Example 3

A series of animal experiments were carried out for examining the preventive effect of the glycoprotein derived from the cancer patient in Example 1 on the immunological rejection in the case of surgical transplantation.

Mice of DKI strain were used in the experiment, and of them, into 15 mice, 20 mg/kg of the glycoprotein was intraperitoneally injected in advance of the transplantation of a piece of skin of C3H/He mouse, and into other 15 mice, 0.2 ml/kg of an aqueous physiological saline solution was intraperitoneally injected as control also in advance of the transplantation.

After the operation of transplantation, the glycoproteins was intraperitoneally injected at a dose of 20 mg/kg/day for 14 days to the experimental mice, and the physiological saline solution was intraperitoneally injected at a dose of 0.2 ml/kg/day for the same number of days as above to control.

No exofoliation of the transplanted piece of skin was observed on the experimental mice administered with the glycoprotein during and even after 2 weeks of the ending of the administration, however, on the other hand, on all the animals of the control group, the transplanted piece of skin exofoliated after 10 days of the transplantation.

Claims

1. A process for the preparation of a glycoprotein having immunosuppressive activity, which process comprises subjecting the serum or the ascites of a human or warm-blooded animal to isoelectric focussing and collecting the fraction at an isoelectric point in the range of 2.6 to 3.6.

2. A process according to claim 1, wherein isoelectric focussing is carried out using a column.

3. A process according to claim 1 wherein isoelectric focussing is carried out using a granular gel.

4. A process according to any one of claims 1 to 3, wherein said serum or said ascites has been collected from a patient suffering from a cancer.

5. A process according to any one of claims 1 to 3, wherein said serum or said ascites has been collected from a warm-blooded animal into which cancer cells or a cancer tissue have been transplanted.

6. A process for the preparation of a glycoprotein having immunosuppressive activity substantially as hereinbefore described in Example 1 or 2.

7. A composition having immunosuppressive activity comprising, as active ingredient, a glycoprotein which has been prepared by a process as claimed in any one of the preceding claims, together with a physiologically acceptable carrier or diluent.

8. A composition according to claim 7 containing the glycoprotein in unit dosage form.

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